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J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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PROVISIONAL SPECIFICATION

Invention Title: "FLAVIVIRUS REPLICON PACKAGING SYSTEM"

The invention is described in the following statement:

TITLE

FLAVIVIRUS REPLICON PACKAGING SYSTEM

FIELD OF THE INVENTION

THIS INVENTION relates to production of virus-like particles of flaviviral origin. More particularly, this invention relates to an inducible flaviviral packaging system that facilitates inducible expression of flaviviral structural proteins necessary for flaviviral RNA packaging in animal cells. In a particular form, the invention provides a tetracycline-inducible packaging system compatible with Kunjin and other flaviviral expression systems that produces unexpectedly high titres of virus-like particles. A particular application of the packaging system is the production of virus-like particles that package RNA comprising a flaviviral replicon and encoding an immunogenic protein or peptide for vaccine delivery.

BACKGROUND OF THE INVENTION

Replicon-based vectors of positive strand RNA viruses have been developed for anti-viral and anti-cancer vaccines (reviewed in Khromykh, 2000. Curr Opin Mol Ther. 2:555-569). Several features make these vectors a desirable choice for development of highly efficient and safe vaccines. These include: (i) high level of expression of encoded heterologous genes (HGs) due to the ability of replicon RNA to amplify itself, (ii) exclusively cytoplasmic replication which eliminates any possible complications associated with nuclear splicing and/or chromosomal integration, (iii) inability of the replicon RNA to escape from transfected (or infected) cell thus limiting the spread of the vaccine vector in the immunized subject which makes these vectors biologically safe, and (iv)

relatively small genome size (7-9 kb) allowing easy manipulations with their cDNA and generation of recombinants.

Replicon-based expression vectors have been developed for representatives of most positive strand RNA virus families, including alphaviruses, picornaviruses, and flaviviruses (reviewed in Khromykh, 2000 *supra*).

In general, VLP delivery has shown to be the most efficient in terms of inducing protective immune responses in mammals.

In particular, expression systems utilizing Kunjin (KUN) flaviviral VLPs have been shown to induce protective immune responses to viral proteins, as described in International Application PCT/AU02/01598.

However, packaging of KUN replicon RNA into VLPs is relatively elaborative and time consuming and requires two consecutive transfections, first with KUN replicon RNA and after a 24-36hr delay with the SFV replicon, RNA expressing KUN structural genes (Khromykh, *et al.*, 1998. J Virol. 72 5967-5977) In addition, the maximum titres of VLPs produced using this system were only about 2 to $5x10^6$ infectious VLPs per ml (Khromych *et al.*, 1998, *supra*; Varnavski & Khromykh, 1999, Virology. 255 366-375) which makes large scale VLP manufacture difficult.

OBJECT OF THE INVENTION

International Publication WO 99/28487 briefly mentions that establishment of a cell line that stably and inducibly expresses flavivirus structural proteins would be a useful approach for the production of VLPs. However, practical implementation of such a system has proven difficult.

In undertaking the establishment of a tetracycline/doxacycline-inducible flavivirus packaging system, the present inventors have unexpectedly shown that

VLP titres produced using an inducible packaging system are at least 100-fold greater than titres typically obtained using prior art packaging systems.

Hence, not only does the inducible packaging system enable single-step transfection with a flavivirus replicon construct for VLP production, but it also results in unexpectedly efficient VLP production.

Furthermore, the inducible flavivirus packaging system may be useful for packaging replicons derived from a variety of flavivirus subgroups.

It is therefore an object of the invention to provide an inducible flavivirus replicon packaging system.

It is another object of the invention to provide a flavivirus replicon packaging system that enhances the efficiency of VLP production.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a plasmid packaging construct for regulatable expression of one or more flavivirus structural proteins in an animal cell, said vector comprising a regulatable promoter operably linked to a nucleotide sequence encoding one or more flavivirus structural proteins.

In another aspect, the invention provides a packaging cell comprising the packaging construct of the first-mentioned aspect.

In yet another aspect, the invention provides a flaviviral expression system comprising:

(i) a plasmid packaging construct for regulatable expression of one or more flavivirus structural proteins in an animal cell, said vector comprising a regulatable promoter operably linked to a nucleotide sequence encoding one or more flavivirus structural proteins; and

(ii) a flaviviral expression construct comprising:

(a) a flaviviral replicon;

(b) a heterelogous nucleic acid; and

(c) a promoter operably linked to said replicon.

In still yet another aspect, the invention provides a host cell comprising the flaviviral expression system of the invention.

In a further aspect, the invention provides a method of producing flavivirus VLPs including the step of:

(i) introducing into an animal cell a plasmid packaging construct for regulatable expression of one or more flavivirus structural proteins in said animal cell, said vector comprising a regulatable promoter operably linked to a nucleotide sequence encoding one or more flavivirus structural proteins;

(ii) introducing into said animal cell a flaviviral expression construct comprising:

(a) a flaviviral replicon;

(b) a heterelogous nucleic acid; and

(c) a promoter operably linked to said replicon; and

(iii) inducing production of one or more VLPs by said packaging cell.

In a still further aspect, the invention provides a method of producing flavivirus VLPs including the step of:

(i) introducing into a packaging cell a flaviviral expression construct comprising:

(a) a flaviviral replicon;

(b) a heterelogous nucleic acid; and

(c) a promoter operably linked to said replicon; and

(ii) inducing production of one or more VLPs by said packaging cell.

Preferably, the flaviviral replicon is of Kunjin virus or Dengue virus origin.

Preferably, the structural proteins are Kunjin virus (KUN) E, prM and C structural proteins.

Preferably, the regulatable promoter is tetracycline inducible.

Throughout this specification, unless otherwise indicated, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of the plasmid constructs used for generation of stable cell lines. pEF-tTA-IRESpuro plasmid was used to generate a first stable BHK cell line, BHK-Tet-Off, continuously expressing tetracycline transactivator (tTA) from the human elongation factor 1α promoter (pEF-1a) (see Results). pTRE2CprME-IRESNeo plasmid DNA was used to generate a second stable BHK cell line, tetKUNCprME, expressing KUN structural genes C, prM, and E (KUN CprME) from tetracycline-inducible CMV promoter (P_{minCMV}). In uninduced tetKUNCprME cells doxycycline (DOX; a form of tetracycline with higher specific activity) binds to tTA and prevents it from binding to tetracycline responsive element (TRE) and subsequent activation of CprME mRNA transcription from CMV promoter. To induce expression of KUN CprME genes, DOX is removed from the medium resulting in the release of tTA, its binding to TRE, and activation of CprME mRNA transcription from CMV promoter. tetR – Tet repressor protein; VP16 – Herpes simplex virus VP16 activation domain; IRES – EMCV internal ribosome entry site; PAC – puromycin N-acetyl

transferase; TRE – Tetracycline-response element; Neo – neomycin resistance gene; SV40 polyA – SV40 transcription terminator/poly(A) signal; β-globin polyA - β-globin transcription terminator/poly(A) signal.

Figure 2. Induction of KUN structural gene expression in tetKUNCprME cells upon removal of doxycycline. (A) Northern blot hybridisation analysis of RNA extracted from induced (-DOX) and uninduced (+DOX) tetKUNCprME and BHK cells. 20 μg of each RNA was separated on a 1% formamide-agarose gel then transferred onto Hybond N membrane by capillary blotting. (B) Western blot analysis of protein extracted from induced (-DOX) and uninduced (+DOX) tetKUNCprME and BHK cells. 5 μg of total protein was separated on a 12.5% polyacrylamide gel then transferred onto Hybond P membrane. The membrane was incubated with KUN anti-E monoclonal antibodies and bound KUN E protein was detected by chemiluminescence.

Figure 3. Fractionation of RNA*gag* VLPs from tetKUNCprME cells in sucrose gradient. 6 ml of 72h culture fluid from tetKUNCprME cells electroporated with KUN*gag* RNA were clarified at 12,000g for 10 min at 4°C. VLPs in the remaining supernatant were precipitated by the addition of polyethylene glycol (MWt 6000) to a final concentration of 6% with continuous rotation at 4°C for 2 h. The precipitated VLPs were then pelleted by centrifugation at 12,000g for 30 min at 4°C. The supernatant was removed, the pellet air dried and resuspended in 300 μl of DMEM overnight on ice at 4°C. The preparation was centrifuged briefly for clarification and then layered onto a 5-25% sucrose gradient. The gradient was then centrifuged at 250,000g for 2.5 h at 4°C. 0.5 ml fractions were collected aseptically from the bottom of the gradient and examined by ELISA for the presence of KUN E protein. Subsequently, peak

fractions were analysed by infection assay for the presence of infectious or non-infectious (empty) KUN virus particles.

Figure 4. Amplification of KUN replicon VLPs in tetKUNCprME cells. Coverslips of tetKUNCprME and BHK21 cells were infected with 0.1 MOI of RNAleuMpt VLPs and analysed by IF with KUN anti-NS3 antibodies at 2d and 3d after infection.

Figure 5. CD8 T cell responses in mice immunised with high titre KUN VLP replicons. Balb/c mice were immunised ip with $2.5x10^7$ pfu KUN VLPs encoding respitary syncytial virus matrix 2 protein (KUN VLP-M2), $2.5x10^7$ pfu KUN VLP not encoding a recombinant antigen (KUN VLP Control), or sc with a peptide vaccine containing the RSV M2 epitope, SYIGSINNI, formulated with tetanus toxoid in Montanide ISA 720 (SYIGSINNI/TT/M720) (n=3 per group). After 15 days splenocytes were removed and analysed for SYIGSINNI-specific responses by (A) IFNγ ELISPOT and (B) by standard chromium release assay (black squares - target cells sensitised with SYIGSINNI peptide. White squares - target cells without peptide).

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have developed a stable packaging construct and packaging cell line tetKUN-CprME that allows simplified (*i.e* one RNA transfection) inducible manufacture of KUN replicon VLPs. In the stable packaging cell line of the invention, KUN structural genes C, prM and E are expressed from the tetracycline-inducible CMV promoter (Fig. 1). During propagation and maintenance of this packaging cell line production of toxic KUN structural gene products is inhibited by addition of tetracycline (or doxycycline) to the medium. The removal of doxycycline from the medium after transfection

of KUN replicon RNA into tetKUN-CME cells results in induction of KUN structural genes expression whose products then package replicating KUN replicon RNA into secreted VLPs (Fig. 1).

Surprisingly, KUN structural proteins produced from this packaging construct of the invention were capable of packaging transfected and self-amplified Kunjin replicon RNA into secreted VLPs at titres of up to ~$4x10^8$ VLPs per ml. This represents ~100 fold improvement over previous packaging protocol employing cytopathic Semliki Forest virus replicon RNA for transient expression of Kunjin structural genes. Secreted KUN replicon VLPs could be harvested continuously three to four times for up to eight days after RNA transfection producing a total amount of up to ~$6.5x10^9$ VLPs from $3x10^6$ transfected cells. Passaging of VLPs on Vero cells and intracerebral injection of VLPs into 2-4 days old suckling mice showed no evidence for the presence of any infectious Kunjin virus in VLP preparations. Immunization of mice with KUN replicon VLPs encoding human respiratory syncytial virus M2 gene induced exceptionally strong CD8+ T cell responses. Packaging cells were also capable of packaging replicon RNA from a distantly related Flavivirus, dengue virus type 2, indicating potential for these cells to package any flavivirus replicon RNA.

It will be appreciated that the present invention may therefore have the following broad applications to flavivirus replicon packaging:

(i) an ability to package any flavivirus replicon; and/or

(ii) an ability to express any flavivirus structural proteins necessary and sufficient for flaviviral replicon packaging.

As used herein, "*flavivirus*" and "*flaviviral*" refer to members of the genus *Flavivirus* within the family *Flaviviridae*, which contains 65 or more related viral

species. Typically, flavivirus are small, enveloped RNA viruses (diameter about 45 nm) with peplomers comprising a single glycoprotein E. Other structural proteins are designated C (core) and M (membrane-like). The single stranded RNA is infectious and typically has a molecular weight of about 4×10^6 with an m7G 'cap' at the 5' end but no poly(A) tract at the 3' end; it functions as the sole messenger. Flaviviruses infect a wide range of vertebrates, and many are transmitted by arthropods such as ticks and mosquitoes, although a separate group of flaviviruses is designated as having no-known-vector (NKV).

Particular, non-limiting examples of flavivirus are West Nile virus, Kunjin virus, Yellow Fever virus, Japanese Encephalitis virus, Dengue virus, Tick-borne encephalitis, Montana Myotis leukoencephalitis virus, Usutu virus, and Alkhurma virus.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA and DNA inclusive of cDNA and genomic DNA.

By "*protein*" is meant an amino acid polymer. Amino acids may include natural (*i.e* genetically encoded), non-natural, D- and L- amino acids as are well known in the art.

A "*peptide*" is a protein having less than fifty (50) amino acids.

A "*polypeptide*" is a protein having fifty (50) or more amino acids.

According to the present invention, a "*packaging construct*" comprises a regulatable promoter operably linked to one or more nucleotide sequences encoding one or more flaviviral structural proteins.

Preferably, the structural proteins are the KUN structural proteins C, prM and E.

However, structural proteins from any other flavivirus may be used. It is well established in the literature that replacement of structural proteins in one flavivirus with those from other flavivirus permits recovery of chimeric flaviviruses (Monath *et al.*, 2000, J. Virol. 74 1742; Guirakhoo *et al.*, 2000, J. Virol. 74 5477; Pletnev *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89 10532) demonstrating that structural proteins from one flavivirus are capable of packaging RNA from another flavivirus.

Additionally, the packaging construct may further comprise other regulatory sequences such as an internal ribosomal entry site (IRES), 3' polyadenylation and transcription terminator sequence (*e.g.* β-globin or SV40-derived) and a selectable marker gene (*e.g.* neomycin, hygromycin or puromycin resistance genes) to facilitate selection of stable transformants.

By "*regulatable promoter*" is meant any promoter operable in an animal cell, wherein promoter activity is controllable in response to one or more regulatory agents. Regulatory agents may be physical (*e.g.* temperature) or may be chemical (*e.g.* steroid hormones, heavy metals, antibiotics).

Examples of such promoters include heat-shock inducible promoters, ecdysone inducible-promoters, tetracycline-inducible/repressible promoters, metallothionine-inducible promoters and mammalian-operable promoters inducible through the bacterial *lac* operon (*e.g.* *lac*-regulated CMV or RSV promoter).

A preferred regulatable promoter is a "tet off" promoter which is repressed in the presence of doxycylcine and induced by removal of doxycycline.

According to a particularly preferred form of this embodiment, the regulatable promoter comprises a CMV promoter linked to a tetracycline response

element (TRE) that facilitates responsiveness to a tetracycline transactivator (tTA) encoded by a separate construct.

According to this preferred form, a stable packaging cell line is developed in two stages:

(i) establishment of a stable cell line expressing tetracycline (doxycycline) transactivator; and

(ii) use of the stable cell line produced in (i) to generate a packaging cell capable of inducibly expressing KUN structural genes after withdrawal of doxycycline.

In this case, the CMV promoter facilitates expression of the operably linked C, prM and E structural genes in the packaging cell. However, it will be appreciated that other promoters may be useful in this regard, such as RSV, SV40, adenoviral and human elongation factor promoters, although without limitation thereto.

By "*operably linked*" or "*operably connected*" is meant that said regulatable promoter is positioned to initiate and regulatably control intracellular transcription of RNA encoding said flaviviral structural proteins.

Suitable host cells for VLP packaging may be any eukaryotic cell line that is competent to effect transcription, translation and any post-transcriptional and/or post-translational processing or modification required for protein expression. Examples of mammalian cells typically used for nucleic acid transfection and protein expression are COS, Vero, CV-1, BHK21, 293, HEK, Chinese Hamster Ovary (CHO) cells, NIH 3T3, Jurkat, WEHI 231, HeLa and B16 melanoma cells without limitation thereto.

Preferably, the host cell is BHK21.

It will be appreciated that packaging cells produced according to the invention may be used for subsequent packaging of flaviviral replicon RNAs encoding one or more proteins.

Flavivirus replicons contemplated by the present invention include any self-replicating component(s) derivable from flavivirus RNA as described for example in International Publication WO 99/28487 and International Application 02/01598. These include without limitation herein DNA-based replicon constructs where replicon cDNA is placed under the control of a mammalian expression promoters such as CMV and delivered in a form of plasmid DNA, and RNA-based replicon constructs where replicon cDNA is placed under the control of a bacteriophage RNA polymerase promoter such as SP6, T7, T3 that allows production of replicon RNA in vitro using corresponding DNA-dependent RNA polymerases and where said replicon RNA can be delivered as naked RNA or as RNA packaged into VLPs.

Although a preferred flaviviral replicon of the invention is derived from Kunjin virus, it will be appreciated by persons skilled in the art that the packaging system of the present invention may be used for packaging any flaviviral replicon.

Examples of flavivirus replicons that are relatively well characterized include replicons from West Nile Virus strains of lineage 1 (Shi *et al.*, Virology, 2002, **296** 219-233) and lineage II (Yamshchikov *et al.*, 2001, Virology, **281** 294-304), dengue virus type 2 (Pang *et al.*, 2001, BMC Microbiology, **1** 18), and yellow fever virus (Molenkamp *et al.*, 2003, J. Virol., 77 1644-1648).

According to the present invention a "*flaviviral expression vector*" comprises a flavivirus replicon together with one or more other regulatory nucleotide sequences. Such regulatory sequences include but are not limited to a

promoter, internal ribosomal entry site (IRES), restriction enzyme site(s) for insertion of one or more heterelogous nucleic acid(s), polyadenylation sequences and other sequences such as an antigenomic sequence of the hepatitis delta virus ribozyme (HDVr) that ensure termination of transcription and precise cleavage of 3' termini, respectively.

Accordingly a "*flaviviral expression construct*" is an expression vector into which a heterologous nucleic acid has been inserted so as to be expressible in the form of RNA and/or as an encoded protein

Said heterologous nucleic acid may encode one or more peptides or polypeptides, or encode a nucleotide sequence substantially identical or substantially complementary to a target sequence.

Heterologous nucleic acids may encode proteins derived or obtained from pathogenic organisms such as viruses, fungi, bacteria, protozoa, invertebrates such as parasitic worms and arthropods or alternatively, may encode mutated, oncogenic or tumour proteins such as tumour antigens, derived or obtained from animals inclusive of animals and humans. Heterologous nucleic acids may also encode synthetic or artificial proteins such immunogenic epitopes constructed to induce immunity.

Introduction of a packaging construct or flavivirus expression construct into an animal cell may be by any method applicable to animal cells. Such methods include calcium phosphate precipitation, electroporation, delivery by lipofectamine, lipofectin and other lipophilic agents, calcium phosphate precipitation, DEAE-Dextran transfection, microparticle bombardment, microinjection and protoplast fusion.

So that the invention may be readily understood and put into practical effect, reference is made to the following non-limiting examples.

EXAMPLES

MATERIALS AND METHODS

Plasmids. The plasmid pEF-tTA-IRESpuro, a derivative of pEFIRES-P (Hobbs *et al.*, 1998 Biochem Biophys Res Commun **252**, 368-72) and containing sequence coding for the tetracycline transactivator (Fig. 1) was a gift from Rick Sturm, University of Queensland). The plasmid pTRE2CprME-IRESNeo (Fig. 1) encoding KUN CprME gene cassette under the control of tatracycline-inducible promoter was constructed as follows. The sequence for the EMCV internal ribosome entry site (IRES) and the neomycin gene were excised from pBS-CIN4IN, a derivative of pCIN1 (Rees *et al.*, 1996, BioTechniques **20** 102-110) using MluI and XbaI. The IRESNeo cassette was then inserted into the corresponding MluI/XbaI sites of pTRE2 vector (Clontech) to produce an intermediate pTRE2IRESNeo plasmid. The sequence coding for the Kunjin (KUN) CprME gene cassette was PCR amplified by high fidelity *Pfu* DNA polymerase (Promega) from FLSDX plasmid DNA template {Khromykh *et al.*, 1998, J. Virol. **72** 5967) using the primers CprMEFor 5'ATTTAGGTGACACTATAGAGTAGTTCGCCTGTGTGA 3' and CprMERev 5'GAGGAGATCTAAGCATGCACGTTCACGGAGAGA 3' to produce a fragment with a BglII restriction enzyme site at the 5' and 3' end. It should be noted that the BglII site at the 5' end of the fragment is located 100 nucleotides downstream of the forward primer and just upstream of the native KUN translation initiation codon. The BglII-BglII fragment containing KUN CprME sequence was then inserted into the BamHI site of pTRE2IRESNeo vector located

upstream of the IRESNeo sequence to produce the pTRE2CprME-IRESNeo plasmid (Fig. 1).

The RNA-based KUN replicon vectors and other KUN replicon constructs encoding different heterologous genes that were used for *in vitro* transcription of different replicon RNAs have been previously (Khromykh & Westaway, 1997, J. Virol. 71 1497; Anraku *et al.*, 2002, J. Virol. 76 3791; Liu, 2002 #1264; Varnavski & Khromykh, 1999, Virology 255 366; Varnavski *et al.*, 2000, J. Virol. 74 4394). KUN replicon encoding M2 gene of respiratory syncytial virus (RSV) was constructed by cloning into RNAleu vector (Anraku *et al.*, 2002, *supra*) of a DNA fragment containing RSV M2 cDNA sequence that was prepared by reverse transcription(RT) and PCR amplification of RNA from RSV-infected cells using appropriate primers. RSV isolate was kindly provided by P. Young, University of Queensland.

The dengue virus type 2 (DEN2) replicon constructs pDENΔCprME and pDENΔprME were derived from the plasmid pDVWS601, which contains a full length cDNA clone corresponding to the genome of the New Guinea C strain of DEN-2 by creating large in frame deletions in the structural genes. pDENΔCprME retained the first 81 nucleotides of the C gene and the last 72 nucleotides of the E gene whilst pDENΔprME retained the first 21 nucleotides of the prM gene and last 72 nts of the E gene.

Cell lines, virus and antibodies. The BHK21 and Vero cell lines were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum and penicillin/streptomycin at 37°C with 5% CO_2. Wild type (wt) KUN virus, strain MRM61C, was grown in Vero cells as described previously (Westaway *et al.*, 1997, J. Virol. 71 6650). Anti-KUN NS3 polyclonal

antibodies raised in rabbits were described previously (Westaway *et al.*, 1997, *supra*). The anti-KUN Envelope 3.91D monoclonal antibody (MAb) raised in mice was a gift from Dr Roy Hall, University of Queensland, Australia (Adams *et al.*, 1995, Virology **206** 49).

DNA transfection. BHK21 cells were cultured for 24 h in a 60 mm dish prior to transfection with 2 μg of plasmid DNA using Lipofectamine Plus reagent (Life Technologies) as described by the manufacturer.

Production of virus-like particles (VLPs) and determination of their titre. KUN replicon RNAs were transcribed in vitro using SP6 RNA polymerase and electroporated into tetKUNCprME cells essentially as described previously (Khromykh & Westaway, 1997, *supra*). Routinely, ~30 μg of RNA were electroporated into 3 x 10^6 cells. The electroporated cells were then seeded into a 100mm dish and incubated in different volumes of medium at 37°C for up to 8 days. Culture fluid (CF) was usually collected at 3-5 time points during this period and replaced with the same volume of fresh medium to allow multiple harvesting of VLPs. The titre of infectious VLPs was determined by infection of Vero cells with 10-fold serial dilutions of the collected CFs and counting the number of cells positive for NS3 expression in IF analysis with anti-NS3 antibodies performed at 30 to 40 h post-infection.

Immunofluorescence. Coverslips of cultured cells were fixed in 4% paraformaldehyde at 28 – 48 hr post-transfection with replicon RNAs or post-infection with VLPs and assayed for expression of KUN NS3 or E protein by indirect immunofluorescence (IF) with anti-NS3 or anti-E antibodies, respectively.

Northern blot analysis. Total RNA was extracted from tetKUNCprME cells cultured with and without doxycycline and from normal BHK21 cells using Trizol reagent (Life Technologies). 20 μg of RNA was separated on a 1% formamide-TAE agarose gel and then transferred to Hybond-N (Amersham-Pharmacia Biotech) by capillary blotting using 20xSSC. An AflII-PstI fragment isolated from pTRE2INeoCprME was used as the template for preparation of labelled probe. This ^{32}P-labelled probe was prepared using the Rediprime II kit (Amersham-Pharmacia Biotech) as described by the manufacturer. The RNA was hybridised with the ^{32}P-labelled DNA probe using ExpressHyb solution (Clontech) at 68°C essentially as described by the manufacturer. Bands were visualised by exposure to X-ray film or by phosphorimaging, and quantitated using the ImageQuant software (Molecular Dynamics).

Western blot analysis. tetKUNCprME cells were cultured for 2 days in a 60 mm dish with and without doxycycline and cellular proteins were extracted using Trizol reagent as described by the manufacturer. BHK21 cell proteins were also recovered for use as a negative control. The protein concentration for each sample was determined using the BioRad Protein assay (BioRad) as described by the manufacturer. Five μg of total cell protein was separated on a 12.5% gel by SDS-PAGE and transferred onto Hybond-P membrane (Amersham-Pharmacia Biotech, UK). The membrane was incubated overnight at 4°C in blocking buffer (5% skim milk/0.1%Tween 20 in phosphate-buffered saline (PBS)). The KUN anti-E MAb was diluted 1:10 in blocking buffer and incubated with the membrane for 2 h at room temperature. The membrane was washed 3 times with 0.1% Tween-20/PBS for 5 min, then the secondary antibody was added. The secondary antibody, goat anti-mouse horseradish peroxidase, was diluted 1:2000 in blocking

buffer and incubated with the membrane for 2 h at RT. The membrane was again washed with 0.1% Tween-20/PBS and developed using the ECL +Plus kit (Amersham-Pharmacia Biotech). The membrane was then exposed to X-ray film for varying time intervals.

RT-PCR and sequencing. Total RNA was extracted from a 60mm dish of tetKUNCprME cells using Trizol. 0.1μg of RNA was reverse-transcribed and amplified using a One-Step RT-PCR kit (Invitrogen). The oligonucleotide primers used were to the KUN cprME region with the forward primer, CoreXbaI 5'GGCTCTAGACCATGTCTAAGAAACCAGGA3' and the reverse primer, cprMERev 5'GAGGAGATCTAAGCATGCCGTTCACGGAGAGA3'. The cDNA product was then used as a template for sequencing with BigDye Terminator Mix (Applied Biosystem) using 6 different primers to cover the full sequence of this region.

KUN E protein detection by indirect ELISA. An indirect ELISA for detection of secreted KUN E present in the culture medium was performed as described in (Adams *et al.*, *supra*).

RESULTS

Establishment of tetracycline-inducible cell line stably expressing KUN structural proteins. Initially, BHK21 cells were transfected with pEF-tTA-IRESpuro plasmid DNA (see Materials and Methods) to establish a BHK cell line stably expressing the tetracycline transactivator. Two days following transfection the antibiotic, puromycin at a concentration of 10 μg/ml, was added for selection of cell clones. Five cell clones were isolated and cultured successfully from this transfection. These clones were then analysed for induction of expression by transfection with the plasmid, pTRE2luciferase (Clontech) in the presence (0.5

μg/ml) or absence of doxycycline. Luciferase expression was determined using the Bright-Glo Luciferase Assay System (Promega). All clones demonstrated varying degrees of induction and background levels (results not shown). Clones #A5 and #A7 were chosen for further studies as they displayed the highest fold induction of luciferase expression compared to uninduced cells.

These two clones were used to establish a stable cell line expressing the KUN structural proteins, i.e., core (C), membrane (prM) and envelope (E). Cells were transfected with pTRE2CprME-IRESNeo plasmid DNA (Fig 1) and subjected to selection with 0.5 mg/ml of Geneticin (G418) in media that also contained 10 μg/ml puromycin and 0.5 μg/ml of doxycycline. Seven cell clones were recovered and cultured for 6 passages. These cell clones were initially screened for their efficiency of packaging KUN replicon RNA into secreted virus-like particles. All seven cell clones were electroporated with KUN replicon RNA (RNAleu; Anraku et al., 2002, J. Virol. 76 3791-2799) to determine whether they were able to produce infectious KUN replicon VLPs. Electroporated cells were then cultured in the medium without doxycyline. The titres of infectious VLPs (in infectious units (IU) per ml) present in harvested culture fluids (CFs) were determined by infection of Vero cells followed by immunofluorescence analysis with anti-NS3 antibodies as described in Materials and Methods. No infectious VLPs were detected in the CFs collected from three of the cell clones. The four remaining clones, #A3, #A8, #E1 and #E5 were capable of VLP production with varying efficiencies (Table 1). Cell clone #A8 was found to be the most efficient cell line for VLP production with the titres of VLPs reaching up to 2×10^8 IU/ml at 53h after electroporation (Table 1). This cell clone was designated tetKUNCprME and used in all further studies.

Characterization of CprME expression and production of secreted replicon VLPs in tetKUNCprME cells. In order to examine the levels of CprME mRNA transcription and intracellular expression of the CprME genes in the tetKUNCprME cell line, cells were cultured for 48 h with and without doxycycline in the media. Normal BHK21 cells were included as a negative control. The CprME mRNA transcription was analysed by Northern blot hybridisation of total cell RNA with a ^{32}P-labelled CprME-specific cDNA probe (Fig. 2A) and the expression of KUN proteins was analysed by Western blot analysis with KUN anti-E antibodies (Fig. 2B). The results showed that there was very little of CprME mRNA and KUN E protein produced in the presence of doxycycline (uninduced cells). In contrast, removal of doxycycline resulted in ~30 fold increase in the level of CprME mRNA, as judged by the relative phosphoimager counts in the corresponding labelled bands (Fig. 2A). Approximately similar increase in the level of KUN E protein production was also detected (Fig. 2B).

To examine levels of secreted KUN proteins and KUN VLPs in the culture fluid of tetKUNCprME cells we initially used indirect ELISA with KUN anti-E antibodies. CFs collected from induced and uninduced tetKUNCprME cells that were cultured for 48 h prior to analysis showed no detectable levels of KUN E protein in both CF samples (Table 2). However, when the same cells were electroporated with RNAleu replicon RNA, a dramatic increase in ELISA readings was noticed by 45 h after RNA electroporation in the CF sample from induced cells, while only a marginal increase in ELISA readings was detected in the CF sample from uninduced cells (Table 2). When VLPs in these CF samples were titrated on Vero cells, the titres of VLPs correlated well with the ELISA

results. 500 IU of VLPs per ml detected in the CF samples collected from uninduced cells produced an ELISA reading OD_{450} of ~0.11, while 2.1 x 10^8 IU of VLPs per ml in the CF sample from induced cells gave an ELISA reading of ~0.63 (Table 2).

The E-antigen positive particles produced in Flavivirus-infected cells and also in our previous preparations of KUN replicon VLPs obtained using the sequential transfection protocol (Khromykh *et al.*, 1998, *supra*) consists of the infectious VLPs containing packaged virion (or replicon) RNA and empty prME particles that do not carry replicating RNA. To differentiate these two forms of particles in the current VLP preparations, CFs from tetKUNCprME cells transfected with KUN*gag* RNA were concentrated by precipitation with PEG6000 and separated on a 5-25% sucrose gradient. Following centrifugation, 0.5 ml fractions were collected from the bottom of the gradient and analysed by ELISA. Two peaks were observed in VLP preparations (Fig. 3, fractions 2-3 and fractions 13-17, respectively) which agrees with our previous results {Khromykh *et al.*, 1998, *supra*). Selected fractions showing the highest and lowest ELISA readings were subjected to infectivity assay on Vero cells. As expected, particles from fractions 2-3 were infectious, while particles from fractions 13-17 were not (Fig. 3).

Optimization of VLP production in tetKUNCprME cells. In order to optimize VLP production, studies were performed with the harvesting of culture fluid and the removal of doxycycline from the media at different time points. Following electroporation of KUN replicon RNA (RNAleu), media containing doxycycline (0.5μg/ml) was incubated on the cells for a further 16 h or 30 h and then fresh media without doxycycline was added. A 60 mm dish of electroporated cells was

set up continually without doxycycline for comparison. The culture fluid was harvested from each dish at 53h and 68h post-electroporation (Table 3). Each sample was examined by infectivity assay on Vero cells. The results showed that the optimal time for induction of CprME expression for VLP assembly is immediately after RNA electroporation (time = 0). A delay in the removal of doxycycline from the media resulted in decrease of VLP production.

We were next interested in determining the optimal VLP harvesting protocol and the ability of tetKUNcprME cells to produce high levels of VLPs encoding various heterologous genes. KUN replicon RNAs encoding different genes such as murine polytope (RNAleuMpt), HIV-1 gag (KUN*gag*), β-galactosidase (repPACβ-gal) and green fluorescence protein (C20DX/GFP/2ArepHDVr) were electroporated into tetKUNCprME cells. VLPs were harvested at different times after RNA electroporations and the medium was replaced with fresh medium every time VLPs were harvested to allow multiple harvesting of VLPs (Table 4). All VLP preparations were then titrated by infection of Vero cells on glass coverslips with serial dilutions of the collected CFs. The average titre of VLPs were in the range of 1×10^7 to 3.8×10^8 IU per ml, and remained high even in the third or fourth consecutive harvest up to 8 days after transfection, depending on the nature of the replicon RNA and the VLP harvesting time (Table 4). The total production of VLPs from the initially transfected 3×10^6 tetKUNCprME cells reached 6.5×10^9 infectious particles for one of the replicons (RNAleuMPt) and was in the range of 1 to 5×10^9 infectious particles for other replicons.

To examine whether KUN replicon VLPs can be amplified in tetKUNCprME but not in normal BHK cells the cells were infected with

RNAleuMpt VLPs at low MOI (0.1) and incubated in the medium without doxycycline for up to 5 days. IF analysis of infected cells with KUN anti-NS3 antibodies showed significant increase in the size of positive cell foci from day 2 to day 3 post-infection (Fig. 4, panels 1 and 2) demonstrating amplification and spread of VLPs in tetKUNCprME cells. In contrast, only individual positive cells were detected in infected normal BHK21 cells at both day 2 and day 3 after VLP infection (Fig. 5, panels 3 and 4). In a separate experiment, an approximately 10-fold increase in VLP titres from day 3 to day 5 of incubation after infection of tetKUNCprME cells with 0.1 MOI of RNAleuMpt VLPs was detected further confirming amplification of VLPs by spread in the packaging cells.

The results convincingly demonstrate that the tetKUNCprME cell-line is able to produce significantly (at least 100-fold) higher titres of KUN replicon VLPs compared to our previously published protocol using cytopathic SFV replicon for expression of KUN structural genes {Varnavski & Khromykh, 1999, *supra*).

Absence of infectious KUN virus in replicon VLP preparations. To confirm that no recombinant KUN virus was produced during production of replicon VLPs in tetKUNCprME cells, CFs harvested at 2 days after transfection with RNAleu RNA were used to infect Vero cells grown on coverslips. The infected cells were incubated for 5 days and examined for expression of E protein by immunofluorescence. The tissue culture fluid from the infected coverslips was then passaged again on fresh cultures of Vero cells for a further 5 days and examined by IF with anti-E antibodies. No E-positive cells were detected in both passages (results not shown). Parallel labelling with anti-NS3 antibodies showed numerous positive cells in the first passage but no positive cells in the second

passage(results not shown) demonstrating that VLPs deliver replicon RNA only in the first round of infection.

Additional evidence of the absence of recombinant KUN virus in VLP preparations was sought by the most sensitive method for virus detection, i.e. intracranial injection of suckling mice. Groups of ten 2-3 day old Balb/C suckling mice were inoculated intracranially with 4 x 10^6 IU of KUN-MPt VLPs or with 1 *pfu* of wt KUN virus (strain MRM61C) as a positive control. All ten mice injected with 1 *pfu* of wt KUN virus developed paralysis of the hindlegs at 4 days post inoculation and had to be sacrificed. In contrast, all VLP-injected mice remained healthy and demonstrated normal development for the duration of the experiment (21 days). These *in vitro* and *in vivo* results clearly demonstrate that production of KUN replicon VLPs in tetKUNCprME cells does not lead to the generation of any recombinant infectious KUN virus.

Immunization with KUN replicon VLPs induces highly potent CD8+ T cell response to an encoded immunogen from the human respiratory syncytial virus. To demonstrate the ability of KUN replicon VLPs produced in tetKUNCprME packaging cells to induce potent antigen-specific immune responses, we gave BALB/c mice a single i.p.immunisation with 2.3x10^7 IU of VLPs containing KUN replicon RNA encoding the respiratory syncytial virus (RSV) M2 gene. Potent CD8 T cell responses specific for the RSV M2 eptope, SYIGSINNI, were generated with ELISPOT analysis showing an average 1400 spots per 10^6 splenocytes (Fig. 5A, KUN VLP M2), and a standard chromium release still showing over 45% specific lysis after effectors were diluted to an effector:target ratio of 2:1 (Fig. 5 B, KUN VLP M2). As expected a control VLP failed to induce significant specific responses, and a peptide vaccine formulated

with SYIGSINNI peptide inducing several fold lower responses (Fig. 5, KUN VLP Control and SYIGSINNI/TT/M720, respectively).

Packaging of dengue virus replicons into secreted infectious VLPs in tetKUNCprME cells. Further examination of this cell line demonstrated that it is capable of packaging other flavivirus replicons such as Dengue. For this, equivalent amounts of *in vitro* transcribed DEN replicon RNAs with a deletion of either CprME (DENΔCME) or ME (DENΔME) were electroporated into tetKUNCprME cells and incubated in the medium without doxycycline. KUN replicon RNA (RNAleu) was included for comparison of VLP production. IF analysis with KUN anti-NS3 antibodies at 2d after transfection showed ~80% and 95% of positive cells after transfection with DENΔME and DENΔCME RNAs, respectively. Transfection of KUN replicon RNA RNAleu resulted in ~95% of NS3-positive cells. KUN NS3 antibodies produced similar results in IF analysis of DEN2 expressed NS3 as DEN2 anti-NS3 antibodies (results not shown). Culture fluid was collected at 48, 72 and 144 h post-electroporation and titrated by infectivity assay on Vero cells. The titre of infectious VLPs produced from DENΔME and DENΔCprME replicon RNAs at 48h after electroporation were 8×10^4 IU/ml and 1.8×10^5 IU/ml respectively. The KUN replicon RNA in the same experiment produced VLPs with a titre of 2.2×10^7 IU/ml. The results demonstrate that DEN2 replicon RNAs could be packaged into secreted VLPs by KUN structural proteins produced in tetKUNCprME cells, albeit with lower efficiency.

Discussion

We have described here a novel packaging system for encapsidation of Flavivirus replicon RNAs into virus-like particles based on the use of

tetracycline-inducible stable packaging cell line tetCprME expressing KUN virus structural genes. Simple VLP production protocol employing only one RNA electroporation, high titres of VLPs reaching up to ~$4x10^8$ VLPs per ml, and multiple VLP harvests for up 8 days allowed a total production of up to ~ $6.5x10^9$ VLPs from a single small scale electroporation. This represents a dramatic improvement over our previously developed KUN replicon packaging system employing cytopathic SFV replicon RNA for transient expression of KUN structural genes {Varnavski & Khromykh, 1999, *supra*; Khromykh *et al.*, 1998, *supra*) and makes feasible a large scale commercial production of KUN replicon VLPs for future vaccine and gene therapy applications. The utility of the KUN replicon VLPs produced in packaging cells for vaccine development was demonstrated by generation in immunized mice of exceptionally strong CD8+ T cell responses to an encoded immunogen from respiratory syncytial virus. In addition, tetCprME cells were also able to package dengue virus replicons into secreted infectious VLPs indicating a possible application of tetCprME cells for production of VLPs encapsidating replicons from other flaviviruses even from distantly related flavivirus subgroups.

Flavivirus structural proteins appear to be one of the primary causes of viral cytopathicity and virus-induced apoptosis (Nunes Duarte dos Santos *et al.*, 2000, Virology 274 292). Low cytopathicity of flavivirus replicons compared to the full-length RNA (Pang *et al.*, 2001, *supra*; Pang *et al.*, 2001, BMC Microbiol. 1 28; Anraku *et al.*, 2002 , *supra*; Khromykh & Westaway, 1997, *supra*; Shi *et al.*, 2002, Virology 296 219; Varnavski & Khromykh 1999, *supra*; Varnavski *et al.*, 2000, *supra*) also demonstrates contribution of structural proteins to viral cytopathicity. Although stable cell lines expressing prM and E cassette from

DEN2 and JE viruses have been generated, the expression levels were low when the native prM-E genes were used (Hunt *et al.*, 2001, J. Virol. Meth. **97** 133). Inactivation of furin cleavage site in prM protein to produce immature prM-E particles with low fusogenic activity (Konishi *et al.*, 2001, J. Virol. **75** 2204), or co-expression of anti-apoptotic bcl-2 gene (Konishi & Fujii, 2002, Vaccine **20** 1058) was required to establish stable cell lines expressing relatively high amounts of prM-E particles. No stable cell lines simultaneously expressing all three Flavivirus structural proteins have been reported to date. We have previously generated Vero cell line stably expressing KUN C protein, however the level of expression was low (Westaway *et al.*, 1997, *supra*). Our attempts previously to generate stable cell line continuously expressing all three KUN structural genes either as continuous gene cassette or under separate promoters using standard DNA expression vectors such as pCI-Neo resulted in either significant reduction of expression or in great instability of expression producing only 10—20% positively expressing cells after a few cell passages (data not shown). Attempts to use these cell lines to produce KUN replicon VLPs resulted in very low VLP titres (data not shown).

Giving the apparent cytotoxicity of the structural proteins and our failure to produce stable cell line using standard expression vectors it was logical to try inducible expression system for generation of packaging cell line. Several inducible systems for regulating transgene activity have been described in many cell types (Rossi & Blau, 1998, Curr. Opin. Biotechnol. **9** 451). Of these, the tetracycline system {Gossen & Bujard, 1992, Proc. Natl. Acad. Sci. USA **89** 5547; Yin *et al.*, 1996, Anal. Biochem. **235** 195) holds the greatest appeal. The system is commercially available from Clontech in Tet-On and Tet-Off

formulations allowing induction of gene expression either by addition or by the removal of tetracycline (http://www.clontech.com/techinfo/manuals/PDF/PT3001-1.pdf). In view of the intended use of KUN replicon VLPs in vaccine applications it was logical to choose Tet-Off system in order to avoid presence of antibiotic in VLP preparations. Approximately 30-fold induction of KUN CprME mRNA transcription and CprME expression was observed in established tetKUNCprME cell line upon removal of doxycyline and the amount of produced KUN structural proteins produced was sufficient to obtain high titres of secreted infectious VLPs after transfection of KUN replicon RNA. The titres of up to ~$4x10^8$ VLPs per ml were obtained which is equal or higher then the viral titres obtained at the peak of KUN virus infection in BHK cells {Khromykh & Westaway, 1994, J. Virol. 68 4580). Importantly, the most sensitive method for detection of KUN virus by intracranial injection of suckling mice clearly showed no infectious KUN virus present in VLP preparations from tetKUNCprME cells. In comparison, BHK packaging cell line expressing Sindbis virus structural protein cassette produced 1-$5x10^8$ of Sindbis or SFV replicon VLPs per ml (Polo et al., 1999, *Proc Natl Acad Sci U S A* **96,** 4598-603). These VLP preparations however, contained ~10^5 *pfu* per ml of infectious recombinant viruses. Splitting the structural proteins into two expression cassettes in the packaging cell line appeared to remove contamination of VLP preparations with infectious viruses to undetectable level, but at the same time reduced the titres of replicon VLPs to $5x10^6$-$1x10^7$ VLPs per ml.

The ultimate test of any vaccine vector candidate is their ability to induce potent immune responses. Our previous studies showed that KUN replicon VLPs

injected at doses up to 10^6 IU per mouse were efficient in induction of reasonably strong immune responses able to protect animals from subsequent experimental viral and tumour challenges {Anraku *et al.*, 2002, *supra*}. Here we show that immunisation of mice with higher doses ($2.5x10^7$ IU) of KUN replicon VLPs encoding the RSV M2 gene that were prepared in tetKUNCprME packaging cells produced exceptionally strong CD8 T cell responses as assessed by ex vivo ELISPOT and chromium release assays. A single vaccination induced responses substantially exceeding those reported following vaccination with recombinant vaccinia virus encoding RSV M2 (Simmons *et al.*, 2001, J. Immunol. **166** 1106) and also exceeding those seen with other immunogens after DNA/poxvirus prime/boost strategies (Schneider *et al.*, 1998, Nat. Med. **4** 397; Woodberry *et al.*, 2003, J. Immunol. **170** 2599.

Although less efficiently, packaging of DEN2 replicon RNA into secreted VLPs was achieved in tetKUNCprME cells. The successful generation of chimeric flaviviruses by replacing structural genes from one virus with those from other flaviviruses (Monath *et al.*, 2000, J. Virol. **74** 1742; Guirakhoo *et al.*, 2000, J. Virol. **74** 5477; Pletnev *et al.*, 1992, Proc. Natl. Acad. Sci. USA **89** 10532) indicate that our results were not unexpected. In our experiments, both DEN2 replicon constructs one with CprME and another one with prME genes deleted were packaged by KUN structural proteins with almost similar efficiency (only ~2-fold difference). In our experiments, both DEN2 replicon constructs (one deleted in CprME genes and another in prME genes) were packaged by KUN structural proteins with almost similar efficiency (only ~2-fold difference). The packaging of DEN2 replicons by KUN structural proteins in tetKUNCprME cells was ~100-fold less efficient then that of KUN replicon. There could be a number

of reasons for this. One of the most likely explanations is that Dengue viruses in general replicate much less efficiently then KUN virus. Previous experiments with full-length infectious DEN2 cDNA showed relatively inefficient production of secreted DEN2 virus directly after RNA transfection into BHK cells (Gualano *et al.*, 1998, *J Gen Virol* 79 437-46). Although we did not compare the efficiencies of replication of DEN2 and KUN replicon RNAs in tetKUNCprME cells, it is likely that replication of DEN2 replicon RNAs would be less efficient then that of KUN replicon RNA and therefore less RNA would be available for packaging.

In summary, the present invention provides a packaging cell line allowing production of large amounts of high-titre secreted KUN replicon virus-like particles free of infectious virus and demonstrated that immunization with these particles induced highly potent immune response to encoded immunogen. The packaging cell line thus should prove to be useful for the manufacture of KUN replicon-based vaccines. In addition, the packaging cell line was also capable of packaging other *Flavivirus* replicons and should prove to be useful in basic studies on *Flavivirus* RNA packaging and virus assembly and in the development of gene expression systems based on different *Flavivirus* replicons. It also suggests that in principle a similar system could be developed for packaging of hepatitis C virus replicons.

Throughout this specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various

modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

The disclosure of each patent and scientific document, computer program and algorithm referred to in this specification is incorporated herein by reference in its entirety.

Table 1. Packaging efficiencies of different tetKUNCprME cell clones.

Cell Clone	VLP titre* (IU/ml)
A3	5.7×10^5
A8	2.1×10^8
E1	2×10^7
E5	5.3×10^4

*2×10^6 cells were electroporated with ~15ug of KUN replicon RNA, RNALeu, and the titres of secreted VLPs harvested at 53h after electroporation were determined by titration on Vero cells.

Table 2. Production of secreted KUN VLPs in tetKUNCprME cells assessed by infectivity assay and by E protein ELISA.

tetKUNCprME cells	ELISA (Abs_{450nm})	VLP Titre (IU/ml)
Uninduced (- RNA)*	0.056	-
Induced (- RNA)*	0.060	-
Uninduced (+RNAleu)	0.114	5×10^2
Induced (+RNAleu)	0.628	2.1×10^8

*For (-RNA) samples cells were maintained for 48h in the medium with (uninduced) or without (induced) 0.5 µg/ml of doxycycline. For (+RNA) samples cells were electroporated with RNAleu RNA and maintained for 48h in the medium with (uninduced) or without (induced) 0.5 µg/ml of doxycycline. The titres of VLPs were determined by infectivity assay on Vero cells. The secreted VLPs were also analysed by E protein ELISA.

Table 3. Effect of CprME expression induction time on VLP production.

Time of induction[a]	VLP production (IU/ml) at hours post electroporation	
	53 h	**68 h**
0 h	2.1×10^8	3×10^7
16 h	< 100	2.9×10^6
30 h	< 100	5×10^5

[a]The induction of CprME expression was initiated by removal of doxycycline at indicated times after electroporation with RNAleu RNA.

Table 4. Production of infectious KUN replicon VLPs encoding different heterologous genes in the tetKUNCprME packaging cell line.

VLP Type	VLP Titre (IU/ml)						Total VLP production
	2d	3d	4d	5d	6d	8d	
RNAleuMPt[a]	3.1×10^7	5.5×10^7	3.8×10^8	-	2.9×10^8	1.3×10^8	6.5×10^9
KUN*gag*[a]	1×10^7	3.9×10^7	1.2×10^8	1.6×10^7	-	-	9.5×10^8
RNAleu[a]	1.8×10^8	1.9×10^8	-	2.5×10^6	-	-	1.65×10^9
repPACβ-gal[b]	4×10^5	-	1.1×10^8	-	2.3×10^8	-	3.4×10^9
C20DX/GFP/2ArepHDVr[c]	1.6×10^8	2.6×10^8	3.7×10^8	2×10^8	-	-	5.2×10^9

3×10^6 cells were electroporated with ~20µg RNA, and VLPs were harvested several times (as indicated) from cells cultivated in 10 cm culture dishes in different volumes of medium. 6ml[a], 10 ml[b], or 5ml[c] of medium in each dish were used for initial VLP harvest and to replace harvested VLPs to allow further VLP production and harvests. Total VLP production was calculated by combining amounts of VLPs obtained in each harvest.

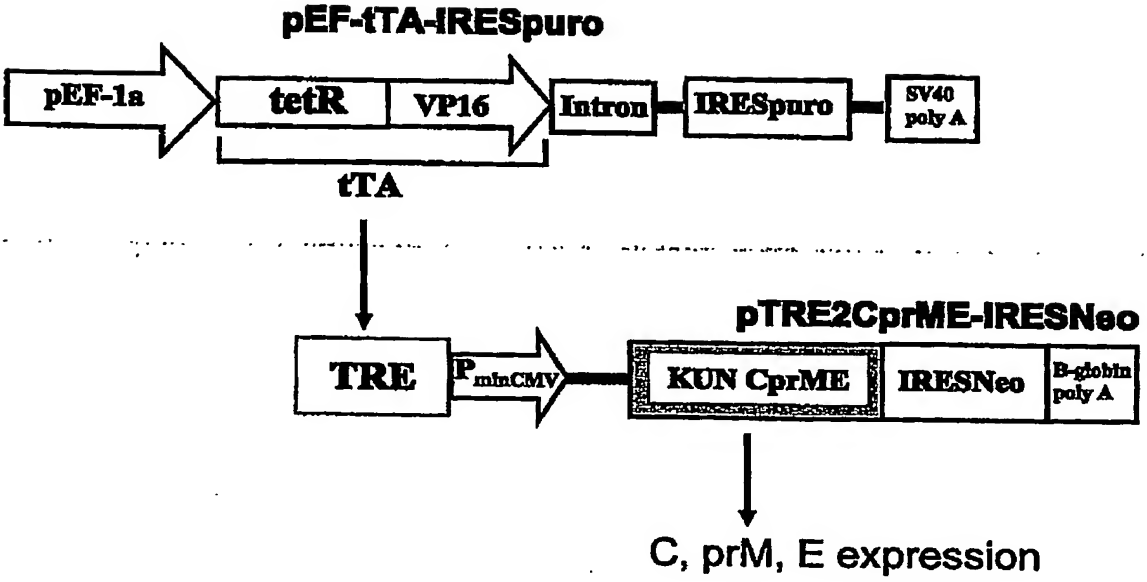
DATED this sixth day of June 2003

THE UNIVERSITY OF QUEENSLAND

by its Patent Attorneys

FISHER ADAMS KELLY

Fig. 1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Intron
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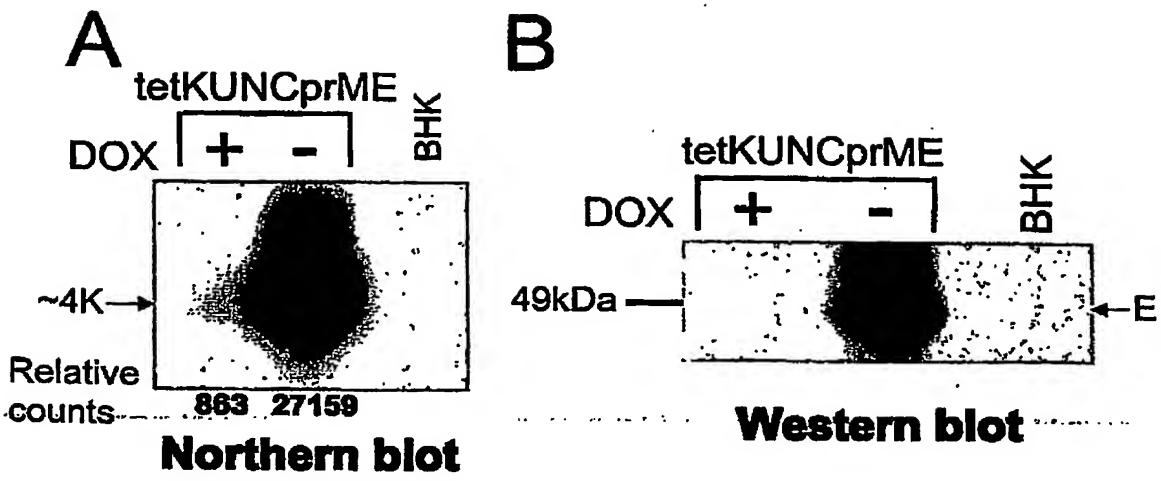
IRESNeo
B-globin poly A
C, prM, E expression

Fig. 2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tetKUNCprME
DOX
+
-
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Relative counts
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BHK
49kDa
E
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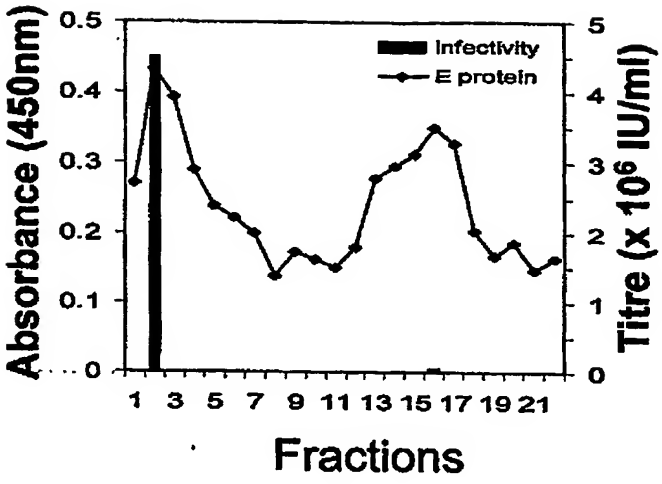
Fig. 3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0.4
0.3
0.2
0.1
0
Infectivity
E protein
5
4
3
2
1
0
Titre (x 10⁶ IU/ml)
1 3 5 7 9 11 13 15 17 19 21
Fractions

Fig. 4

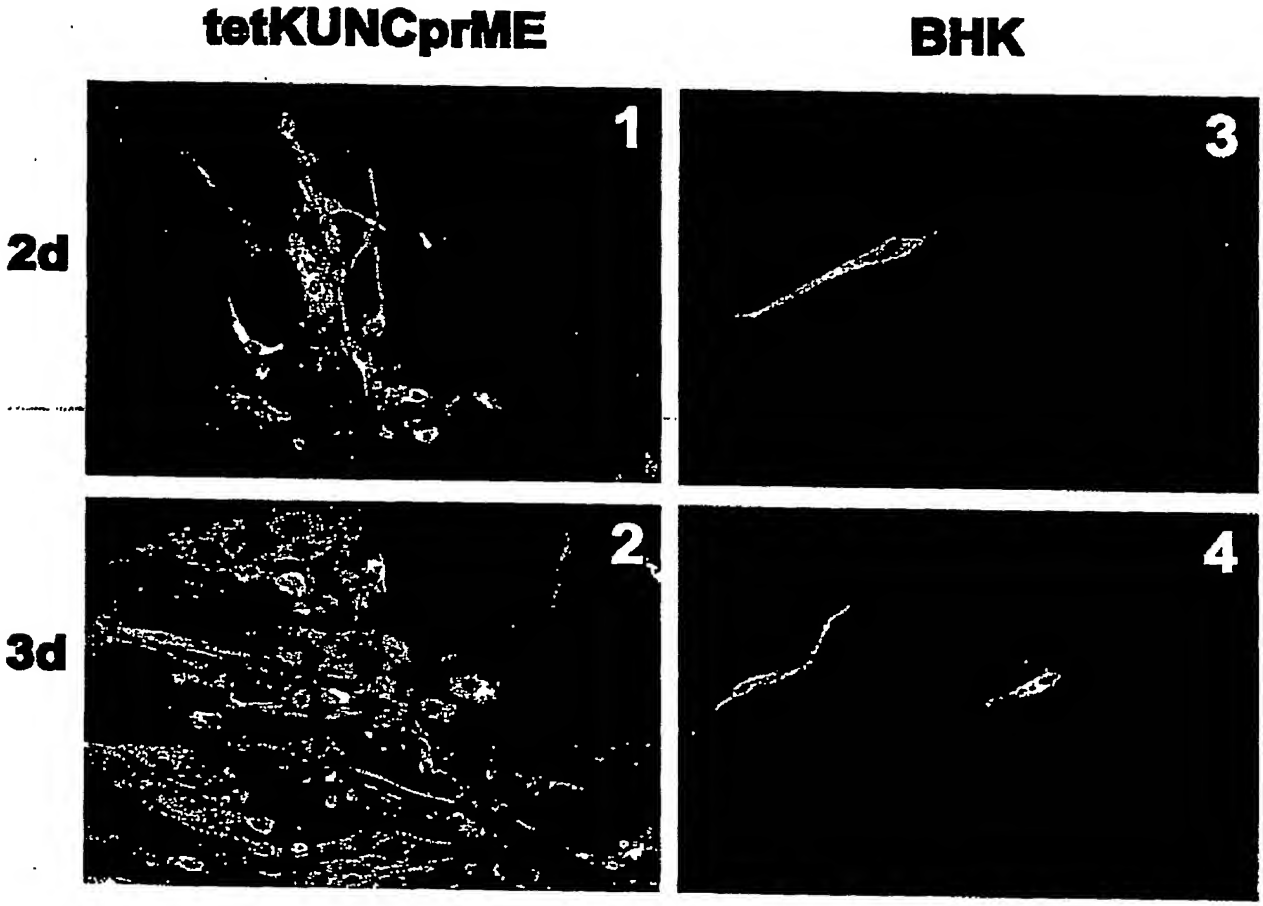
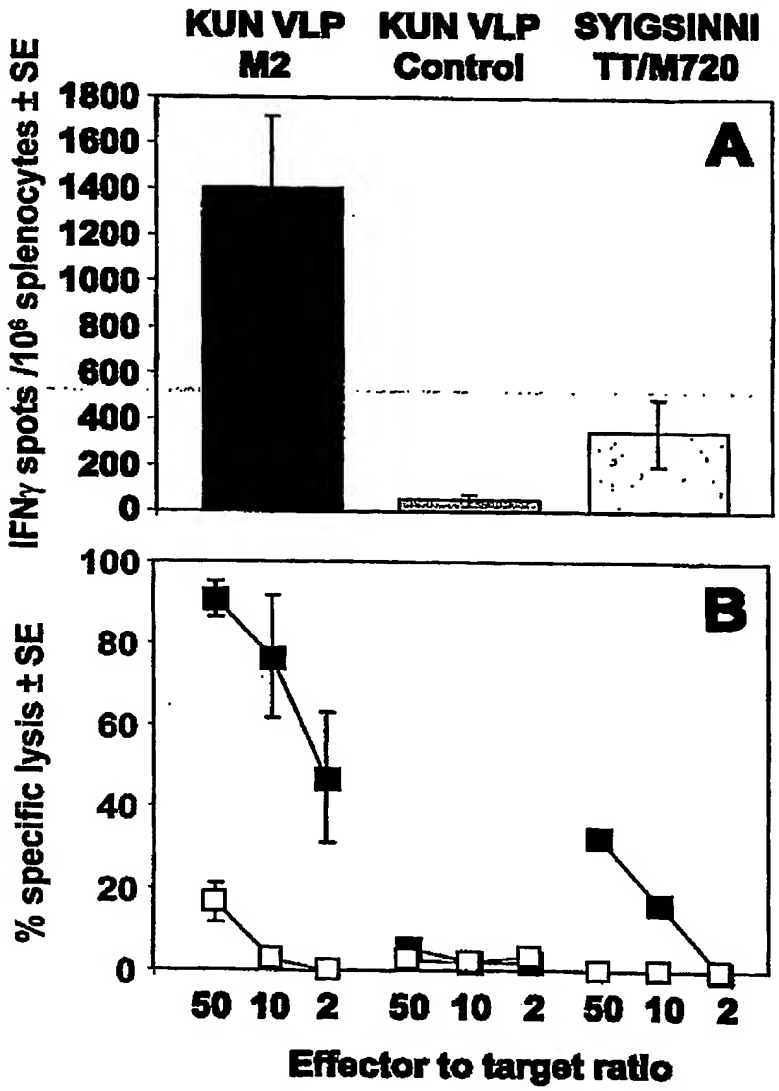


Figure 5


KUN VLP M2
KUN VLP Control
SYIGSINNI TT/M720
IFNγ spots /10⁶ splenocytes ± SE
1800
1600
1400
1200
1000
800
600
400
200
0
A
% specific lysis ± SE
100
80
60
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B
50 10 2 50 10 2 50 10 2
Effector to target ratio